Development and Validation of Radiation-Responsive Protein Bioassays for Biodosimetry Applications

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ABSTRACT

The present need to assess radiation dose rapidly in mass-casualty and population-monitoring scenarios prompted an evaluation of potential protein biomarkers that can provide early diagnostic information after exposure. Using an ex vivo model system of human peripheral lymphocytes as well as an in vivo murine model, we demonstrated radiation-responsive changes in the expression of the proto-oncogene proteins ras-p21, raf-1, and DNA repair protein p21Waf1Cip, each with a progressive time- and radiation-dose-dependent increase. In addition, we adopted a methodology to identify, optimize, and validate radiation-responsive molecular biomarkers that employs Luminex™ technology, a microsphere-based multi-analyte assay system. This technology is based on the principles of the sandwich immunoassay and flow-cytometric analysis in a 96-well microtiter plate format. Current studies use reagents prepared in-house by conjugating capture antibodies to Luminex™ microspheres, and biotin to detection antibodies. Preliminary results demonstrate that radiation-responsive changes in the level of GADD45 DNA repair protein occurred with a progressive time- and radiation-dose-dependent increase in the range of 0.15 to 6.0 Gy. A robotic analysis system for processing the blood protein bioassay was established at the Armed Forces Radiobiology Research Institute (AFRRI). The system consists of a Qiagen Biorobot-8000 for large-sample liquid handling to work in concert with the Luminex™ platform. This robotic system provides "proof of concept" for high-throughput isolation, detection, and quantification of blood protein biomarkers for radiation-dose assessment. Use of this sandwich immunoassay bioassay approach is compatible with field-deployable and hand-held diagnostic platforms. Use of validated molecular biomarker assays linked with existing AFRRI medical recording software and medical data-recording forms, available at the website www.afrri.usuhs.mil, would provide enhanced tools for the medical community to effectively manage a radiation casualty incident.

1.0 INTRODUCTION

In 1995, the U.S. military requested the Medical Follow-up Agency of the Institute of Medicine to provide advice related to exposure of military personnel to radiation doses. Dr. F.A. Mettler (University of New Mexico, Albuquerque, NM) chaired a report of the Committee on Battlefield Radiation Exposure Criteria, which recommended establishing a program of individual measurement, recording, maintenance, and use of dosimetry and exposure information [Institute of Medicine 1999]. Personnel physical dosimeters are recommended and advocated for use by radiation workers, astronauts [Semkova 1995; Apathy 1999], and U.S. military personnel at risk of exposure to ionizing radiation. Although physical dosimeters of radiation expo-

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sure are useful for radiation-protection applications, their use alone is considered inadequate to provide necessary diagnostic information to treat individuals for life-threatening radiation exposures. Changes in the physical location of dosimeters on individuals, whole-body versus partial-body exposures, and variation in dose rate all affect the determination of radiation dose exposure and introduce significant uncertainties in estimating radiation injury that require medical treatment and care. Biological dosimetry assay systems complement physical dosimetry because they weigh the different components of the radiation environment according to their biological efficacy [Horneck 1998].

In 1996 and 1999, scientific sessions at conferences sponsored by the Armed Forces Radiobiology Research Institute (AFRRI) evaluated candidate biodosimetric approaches useful in military operations [Blakely 1998; Blakely 2002b]. In addition, the Defense Threat Reduction Agency in 2001 sponsored a conference held by the National Council of Radiation Protection and Measurements (NCRP) on current available and emerging technologies to provide biodosimetry capability in military operations [Preston 2001]. In October 2004, the Council of Ionizing Radiation Measurements and Standards focused their annual meeting on biological dosimetry measurements and standards. The consensus from these national and international conferences was the use of a multiple dosimetry approach involving radiation dosimeters, radioactivity measurements, and biological assays for radiation exposure assessment related to medical radiological management applications.

The use of multiple bioassays for dose assessment represents the current state of art in radiation accident biodosimetry for definitive dose assessment [Blakely 2002a; Blakely 2002d]; however, current gaps exist in an effective dosimetry system for operational military medical applications. Recognizing the need to fill these gaps, the U.S. Army has identified several high-priority specific military requirements (SMR) for radiation and nuclear weapons effects including: a) low-level radiation individual digital dosimeter (SMR rank 8), b) field radiological biodosimetry (SMR rank 12), c) non-invasive field biodosimetry (SMR rank 15), and d) deployable dosimetry system (SMR rank 16) [USANCA, 2003]. Advances in the development, validation, and fielding of radiological countermeasures in this area also would have direct relevance to medical preparedness for emerging risks associated with radiological terrorism [Yehezkelli 2002].

Molecular biomarkers are used as diagnostic endpoints in environmental health [Vainio 2001] and cancer [Preston 2002] risk assessments. For example, the blood level of prostate-specific antigen is a predictive indicator for prostate cancer risk. The human genome has some 50,000 to 100,000 genes that represent the template for many more proteins, generally with proteomic patterns specific to cell types and tissues. Biological monitoring of molecular biomarkers can provide radiation exposure assessment [Horneck, 1998; Becciolini 2001; Blakely 2001; Blakely 2002c, Blakely 2002d]. Although still in its infancy as a scientific discipline, the study of radiation biomarkers could include DNA mutations, gene expression, and protein endpoints. Cellular responses to ionizing radiation have been evaluated using gene-expression array technologies. A few highly over-expressing sentinel radiation-responsive targets were identified from an array of distinct gene-expression profile responses [Amundson 2000; Amundson 2002].

There also are efforts to identify candidate radiation-responsive protein biomarkers (Table 1). Hofmann and colleagues reported radiation-induced increases of serum amylase in 41 patients, following either whole-body irradiation or irradiation of the head and neck region [Hofmann 1990]. Becciolini and colleagues advocate the use of biochemical (e.g., serum amylase and tissue polypeptide antigen) dosimetry for prolonged spaceflights [Becciolini 2001]. Low doses of radiation, in the range commonly received by atomic radiation workers or as a result of minor medical diagnostic procedures (0.25 to 10 mGy), stimulate the expression of IL-2 receptors (IL-2R) on the surface of peripheral blood lymphocytes (PBL) taken from normal human donors [Xu 1996]. Additional studies are needed to validate candidate protein biomarkers for applied biological dosimetry appli-

cations.

Table 1: Selected list of radiation-responsive protein biomarkers and their respective tissue or cell location

Protein	Tissue or cell location	References
Amylase, tissue polypeptide antigen	Parotid gland	[Hofmann 1990; Dubray 1992; Becciolini 2001]
Cytokines (IL-6, TNF-α)	Skin and blood	[Beetz 1997]
Alkaline phosphatase; Gadd45	Blood	[Papathanasiou 1991; Donnadieu-Claraz 1989]

Diagnostic assessment of radiation exposure is necessary to support triage of radiation casualties and develop treatment strategies for individuals exposed to life-threatening injuries. No single biodosimetric assay is adequate to provide medical response for varied radiation-exposure scenarios as well as to provide surge-response capabilities due to mass radiological casualties [Prasanna 2004]. A multiple parameter biodosimetry system should be developed using diagnostic equipment with dual-use application (e.g., general medical care). Here we describe research findings of AFRRI's Biological Dosimetry Team members identifying, optimizing detection, and validating protein biomarkers for radiation dose assessment. These studies were designed to contribute toward the development of multiple parameter bioassays (signs/symptoms, hematology, cytogenetic, and molecular biomarkers) and the use of software applications to record and integrate multiple biodosimetry diagnostic information for medical management of radiation casualties [Sine 2001; Salter 2004].

2.0 RESULTS AND DISCUSSION

2.1 *Ex Vivo* Human Peripheral Blood Lymphocyte Radiation Model Studies

At AFRRI, initial radiation protein biomarker studies focused on detection of proto-oncogene protein products following *ex vivo* exposure of isolated human peripheral blood lymphocytes (HPBL) to ionizing radiation. The experimental protocol for isolating HPBL and exposing these cells to ionizing radiation was similar to that previously described [Miller 2002] and were modeled after parallel studies evaluating radiation-induced chromosome aberrations [Prasanna 2002]. Proto-oncogenes (i.e., ras p21, raf) in the cell pellets were measured using conventional dual-antibody, enzyme-linked, immunosorbent serologic assay (ELISA) methodology [Brandt-Rauf 1998]. Figure 1 illustrates the time course for ras p21 and raf proteins detected in lymphocyte cell pellets after exposure to 10 and 75 cGy x-rays, showing the potential utility of protein biomarkers to detect radiation exposure.

2.2 *In Vivo* Murine Blood Serum Radiation Model Studies

The *ex vivo* HPBL protein radiation biomarker findings at AFRRI catalyzed the initiation of radiation protein biomarker studies using an *in vivo* murine radiation model. Male BALB/c mice were exposed to 25-cGy ^{60}Co-gamma radiation. Dosimetry was performed as previously described [Miller 1999]. Serum levels of proteins were measured at various times following radiation (<120 hr) by conventional ELISA assays.

Exposure of mice to 25 cGy gamma rays resulted in the up-regulation of serum levels of apoptosis (Bax), anti-apoptosis (Bcl2), proto-oncogene (ras-p21), and DNA repair (p21Waf1Cip1) proteins (Figure 2) [Miller 1999;

Blakely 2003a]. The use of multiple protein targets were evaluated in order to provide additional radiation specificity and sensitivity. Results from these preliminary *in vivo* validation studies established an initial proof-of-concept that radiation biomarkers could provide diagnostic information useful for radiation-exposure assessment.

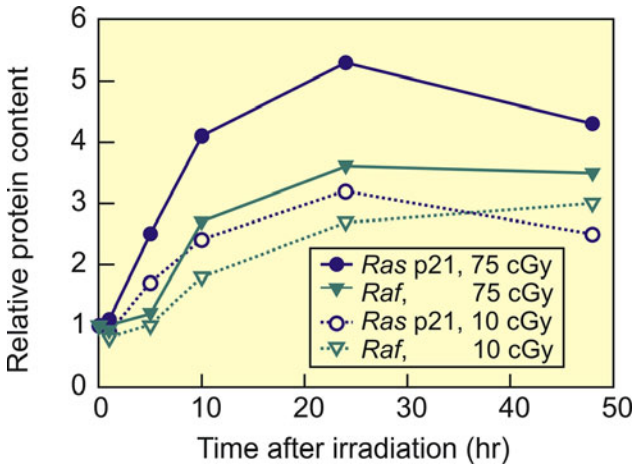

Figure 1: Time course of ras p21 and raf protein content in human *ex vivo* blood lymphocyte model after exposure to 10- and 75-cGy 250 kVp x-ray exposure (100 cGy/min). Samples are derived from the blood lymphocyte cell pellets. Protein content is determined by spectrophotometric analysis. Specific protein biomarkers were detected at equivalent total protein levels using a conventional ELISA method. Symbols represent the means (n = 5, SE were <20% of the means) for ras p21 (circles) and raf (triangle) after exposure to 10 (open circles) and 75 (solid circles) cGy. Components of these results are derived from previous published studies [Miller 1999; Blakely 2003b].

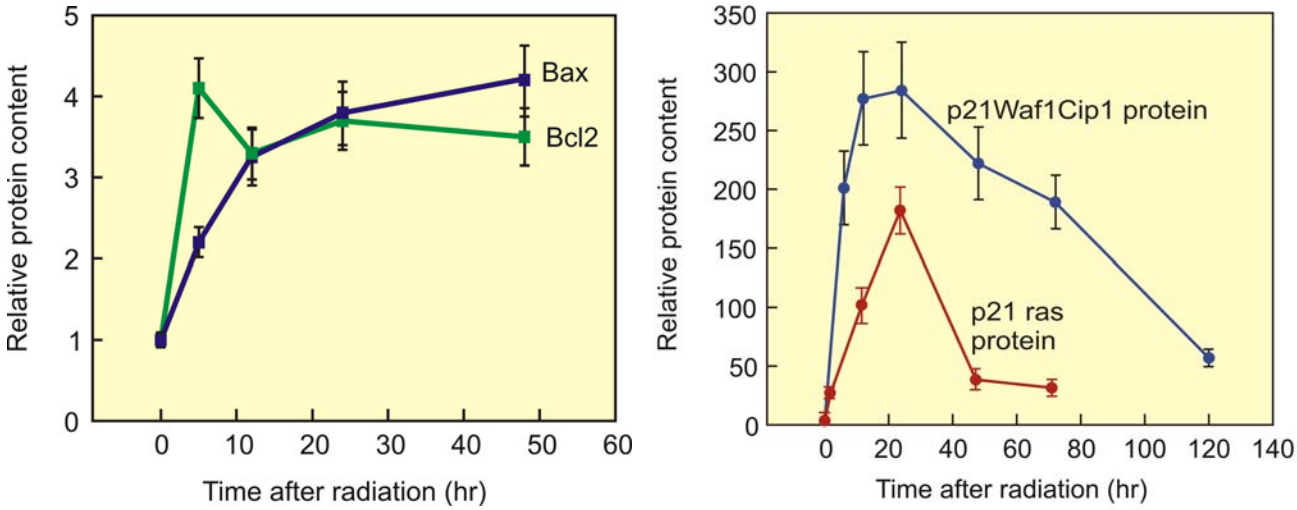

Figure 2: Radiation-responsive changes in the expression of ras-p21, p21 Waf1Cip1, Bax, and Bcl2 in peripheral blood serum of 25-cGy irradiated rodents. See manuscript text for additional experimental details. Each symbol represents the mean from 5 to 12 animals. Components of these results are derived from previous published studies [Miller 1999; Blakely 2003a].

2.3 Microsphere Immunoassay Studies Using *In Vitro* Human Blood Radiation Model

2.3.1 Microsphere Immunoassay

Although the dual antibody sandwich ELISA is a convenient and adequate procedure to quantify protein biomarkers, it has some significant limitations. The assay requires high-sample volume and cannot be multiplexed. We have initiated a strategy to optimize and validate radiation-responsive protein biomarkers using a microsphere-based multi-analyte assay system (Luminex-100™). The approach of using the microsphere-based capture sandwich immunoassay was highly appealing because this technology demonstrates cost-effectiveness for multiplexing capability and high-sample throughput analysis. This technology is based on microscopic spherical polystyrol particles that serve as a solid phase for molecular detection reactions [McHugh 1994]. Labelled particles are then measured using a flow cytometer equipped with a 96-well microtiter plate platform. The method used a mixture of two distinct sets of uniquely fluorescent micro-spheres, i.e., an array of fluorescent micro-spheres [Kettman 1998], which were identified by distinct red and orange fluorescent internal dyes by the Luminex-100™ flow analyzer [Fulton 1997]. Quantification was accomplished with a green fluorescent reporter molecule. At present, 100 distinct sets of fluorescent micro-spheres are available, permitting multiple detection reactions to be carried out simultaneously in very small sample volumes. Furthermore, this technology demonstrates unsurpassed sensitivity, specificity, high-throughput potential and flexibility. In these initial studies we selected the radiation-responsive GADD45 protein target due to the robust dose responses demonstrated using gene-expression bioassay with a human blood *ex vivo* radiation model (Grace 2002). Luminex™ reagents for detecting the DNA strand break-repair protein, GADD45α, were prepared using protocols provided by the manufacturer (Luminex Corp., Austin, TX). A calibration curve (data not shown) was obtained using a GADD45α protein standard (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

2.3.2 Radiation dose response using *ex vivo* human blood

These Luminex™ studies at AFRRI used a human peripheral blood model exposed *ex vivo* to ^{60}Co gamma rays (0–6 Gy) at dose rates of 10 cGy/min. The experimental protocol for blood collection and exposing blood cells to ionizing radiation was modeled after parallel studies evaluating radiation-induced gene expression [Blakely 2002; Grace 2002]. GADD45α protein was detected in cell pellets using the Luminex™ methodology at 24 and 48 hr after radiation. Radiation caused a progressive dose dependent increase in GADD45α protein in blood cell pellets (Figure 3) and blood serum (data not shown). A similar radiation dose-dependent increase in GADD45α protein was observed in another experiment (0-3 Gy) using blood serum

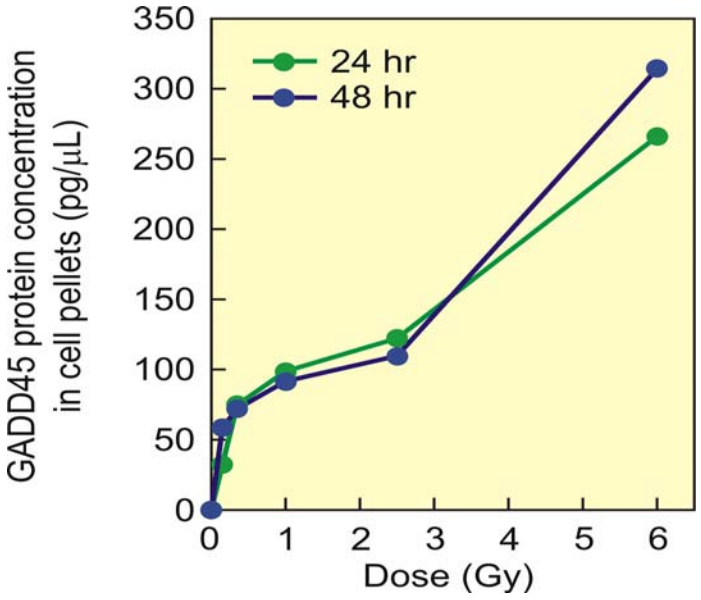

Figure 3: Radiation-responsive changes in the GADD45α levels in human blood cells at 24- and 48 hr after exposure to exposure to ^{60}Co gamma rays (10 cGy/min). See manuscript text for additional experimental details. Each symbol represents the mean of two replicates from a single experiment.

(data not shown).

2.3.3 Simulated Partial-Body Radiation Exposure Human Blood Study

Radiation accidents typically involve partial-body exposures. In order to evaluate the potential utility of the candidate radiation protein bioassays for this radiation scenario, we performed a simulated *ex vivo* partial-body exposure study similar to that described previously using a chromosome aberration endpoint [Blakely 1995]. Table 2 illustrates the experimental design involving exposing blood samples to 6 Gy ^{60}Co gamma rays (10 cGy/min) and mixing with control (sham) sample immediately after exposure. The mixtures were then incubated for 24 hr and GADD45α protein detected in blood cell pellets using another batch of GADD45 Luminex™ reagents. These results are consistent with the GADD45α data described earlier and suggest that the GADD45 sandwich immunoassay can predict partial-body exposures (Figure 4).

Table 2: Experimental design for simulated partial-body exposure model using *ex vivo* irradiated human blood

Percent blood irradiated	Blood sample mixture	
	0-Gy sample, %	6-Gy sample, %
0	100	0
20	80	20
40	60	40
60	40	60
80	20	80
100	0	100

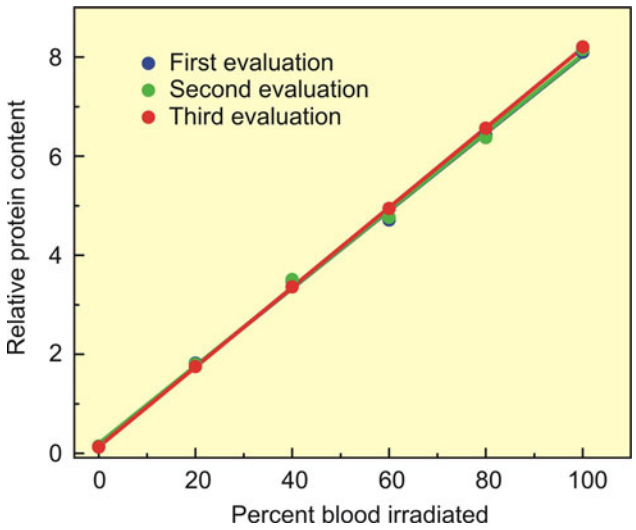

Figure 4: GADD45α protein content as a function of percent human blood irradiated in a simulated partial-body exposure. *Ex vivo* human whole blood was exposed to 6 Gy (10 rads/min) of bilateral radiation and mixed with non-irradiated blood from the same donor. The mixed blood was incubated with RPMI plus 10% fetal bovine serum media and cultured for 24 hr. The simulated partial-body exposure was analyzed via Luminex™ technology. Three independent experiments were performed. The symbols represent the means of each replicate experiment. Standard errors of the means are less than the symbol size.

2.4 Analysis Platforms

2.4.1 Reference Clinical Laboratory System

A robotic analysis system for processing the blood protein microsphere immunoassay was designed and established at AFRRI. The system consists of a robotic liquid-handling system (Qiagen Biorobot 8000) designed specially for the microsphere immunoassay, a UV/VIS spectrometer (PowerWave X Spectrophotometer) compatible to perform total protein-quantification analysis of samples on microplates, and a dedicated flow cytometry system (Luminex™-100) for analysis of blood proteins using the microsphere-based immunoassay (Figure 5).

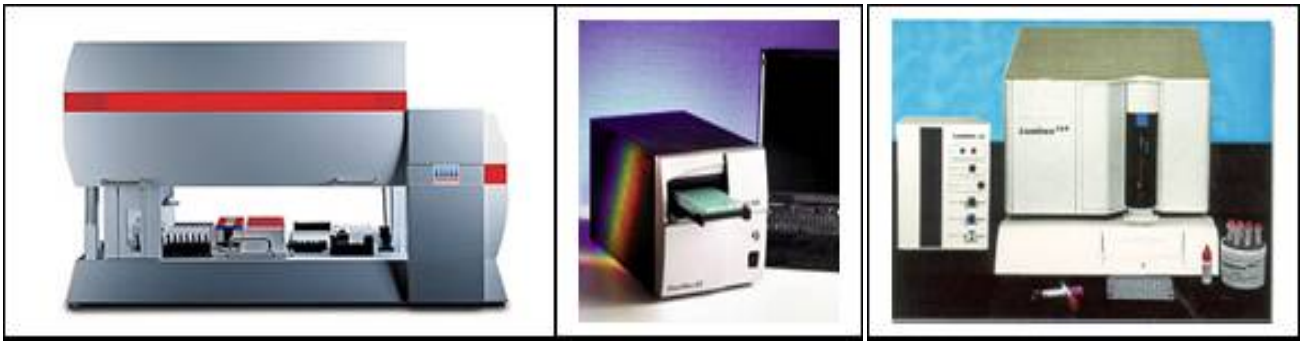

Figure 5: AFRRI's high-throughput microsphere immunoassay analysis system. The photographic images show the analytical system that robotically processes blood samples to measure protein biomarker changes predictive of radiation exposure using the microsphere immunoassay. See manuscript text for details. Components of this system were derived from a previous published study [Blakely 2003b].

The Qiagen BioRobot 8000 currently functions using pre-installed generic liquid-handling protocols. The UV/VIS spectrophotometer plate reader with the microplate format enhances our ability to quantify total protein. These total protein data are essential for selecting the volume of sample necessary for the sandwich immunoassay performed on the Luminex™ -100 instrument. In conjunction with the Luminex™ instrument, the BioRobot 8000 provides the liquid-handling automation required for high-throughput analysis of samples for the protein biomarker(s) of interest.

2.4.2 Field-Deployable Laboratory and Hand-Held Analytical Systems

The U.S. Army currently uses conventional ELISA-based diagnostic analysis platforms in their field deployable laboratory teams (i.e., the 1st and 9th Area Medical Laboratories). Advanced analytical systems using technologies based on molecular biology have been adopted by the U.S. military for deployable field laboratory applications of biological pathogen detection [Belgrader 1999]. Further miniaturization of diagnostic equipment used to detect nucleic acid sequences [Northrup 1998; Anderson 2000] and antigen-based biomarkers would enhance diagnostic capabilities in field operations. Exploitation of these analytical instrument systems for diagnostic biodosimetry applications has significant military operational benefits [Blakely 2002b].

3.0 SUMMARY

- Established proof-of-concept data, based on *in vitro* HPBL and *in vivo* murine blood serum studies, showing that blood protein biomarkers are a potentially useful diagnostic biomarker for radiation exposure assessment.
- Characterized preliminary radioresponse for GADD45α using *ex vivo* human blood model systems. Overall, these GADD45 radioresponse results using the microsphere immunoassay demonstrate that our approach is a feasible, high-throughput, and rapid biodosimetry approach for radiation dose assessment.
- Efforts currently underway are to further optimize a high-throughput robotic system and to collaborate with others to provide additional *in vivo* validation data of radiation-responsive protein biomarkers for radiation injury and dose assessment.

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